Effect of TSHAC on Human Cytochrome P450 Activity, and Transport Mediated by P-Glycoprotein

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TSAHC [4′-(p-toluenesulfonylamido)-4-hydroxychalcone] is a promising antitumorigenic chalcone compound, especially against TM4SF5 (four-transmembrane L6 family member 5)-mediated hepatocarcinoma. We evaluated the potential of TSAHC to inhibit the catalytic activities of nine cytochrome P450 isoforms and of P-glycoprotein (P-gp). The abilities of TSAHC to inhibit phenacetin O-deethylation (CYP1A2), coumarin 6-hydroxylation (CYP2A6), bupropion hydroxylation (CYP2B6), amodiaquine N-deethylation (CYP2C8), diclofenac 4-hydroxylation (CYP2C9), omeprazole 5-hydroxylation (CYP2C19), dextromethorphan O-demethylation (CYP2D6), chlorzoxazone 6-hydroxylation (CYP2E1), and midazolam 1′-hydroxylation (CYP3A) were tested using human liver microsomes. The P-gp inhibitory effect of TSAHC was assessed by [3H]digoxin accumulation in the LLCPK1-MDR1 cell system. TSAHC strongly inhibited CYP2C8, CYP2C9, and CYP2C19 isoform activities with Ki values of 0.81, 0.076, and 3.45 µM, respectively. It also enhanced digoxin accumulation in a dose-dependent manner in the LLCPK1-MDR1 cells. These findings indicate that TSAHC has the potential to inhibit CYP2C isoforms and P-gp activities in vitro. TSAHC might be used as a nonspecific inhibitor of CYP2C isoforms based on its negligible inhibitory effect on other P450 isoforms such as CYP1A2, CYP2A6, CYP2B6, CYP2D6, CYP2E1, and CYP3A.

Keywords: TSAHC, cytochrome P450s, P-glycoprotein, human liver microsomes, drug interactions
TSAHC on P-glycoprotein (P-gp) activity. Such information may be of considerable clinical impact in terms of avoiding potential drug interactions.

**Materials and Methods**

**Chemicals and Reagents**

4'-(p-Toluenesulfonylamido)-4-hydroxychalcone (TSAHC) was synthesized by Gyeongsang National University (Inju, Korea) with a purity >99.0% (Fig. 1). Phenacetin, acetaminophen, coumarin, 7-hydroxycoumarin, bupropion, amodiaquine, diclofenac, omeprazole, dextromethorphan, dextorphan, chlorzoxazone, chlorpropanolide, β-nicotinamide adenine dinucleotide phosphate (NADP), magneesium chloride, glucose-6-phosphate (G6P), glucose-6-phosphate dehydrogenase (G6PDH), cyclosporine A (CsA), and para-aminobipiperlic acid (PAH) were purchased from Sigma-Aldrich (St. Louis, MO, USA). N-Desethylamodiaquine, 4-hydroxydiclofenac, 5-hydroxysomeprazole, 6-hydroxychlorzoxazone, midazolam, and 1'-hydroxymidazolam were obtained from Toronto Research Chemical (Toronto, Canada). [3H]Digoxin (9.6 Ci/mmol) was purchased from Perkin Elmer Inc. (Boston, MA, USA). Pooled human liver microsomes and 4-hydroxybupropion were purchased from BD Gentest (H161, 5 mg/ml, Woburn, MA, USA). Solvents were HPLC grade (Fischer Scientific Co., Pittsburgh, PA, USA) and the other chemicals were of the highest quality available.

**Inhibitory Potency of TSAHC on P-gp-mediated Digoxin Efflux**

LLCPK1-MDR1 cells, obtained from Dr. A. H. Schinkel (Netherlands Center Institute, Amsterdam, The Netherlands), were grown in tissue culture flasks in DMEM supplemented with 10% fetal bovine serum, 1% nonessential amino acids, 4 mM L-glutamine, and 1% penicillin-streptomycin. The cells were maintained at 37°C in a humidified atmosphere with 5% CO2/95% air. The P-gp inhibitory effect of TSAHC was assessed by digoxin efflux using LLCPK1-MDR1 cells (10^5 cells/well, 96-well plate). After the cells reached 95% confluence, the growth medium was discarded and the attached cells were washed and pre-incubated with Dulbecco’s phosphate buffered saline (DPBS) supplemented with 20 mM glucose, 9 mM sodium bicarbonate, and 25 mM HEPES for 30 min at 37°C. The reaction was initiated by adding a NADPH-generating system (1.3 mM NADP, 3.3 mM G6P, 3.3 mM MgCl2, and 500 unit/ml G6PDH). Incubations were performed for 15 min at 37°C in a shaking water bath. The substrates were used at concentrations approximately equal to their respective Ks values: 50 µM for phenacetin, 5 µM for coumarin, 50 µM for bupropion, 5 µM for dextromethorphan, 50 µM for chlorzoxazone, and 5 µM for midazolam. All incubations were performed in triplicate, and mean values were used for analysis.

**Assay of Amodiaquine N-Deethylase, Diclofenac 4-Hydroxylase, and Omeprazole 5-Hydroxylase Activities of Human CYP2C Isoforms**

Assays for amodiaquine N-deethylase, diclofenac hydroxylase, and omeprazole 5-hydroxylase activities of human CYP2C8, CYP2C9, and CYP2C19, respectively, were performed according to the methods of Dravid and Frye [3], Ji et al. [10], and He et al. [9], with minor modifications. Briefly, each incubation was performed with pooled human liver microsomes (Gentest H161, 0.25 mg/ml) in 100 mM phosphate buffer (pH 7.4) in a final incubation volume of 100 µl. The incubation mixtures, each containing one of the CYP2C isofrom-specific substrates (amodiaquine for CYP2C8, diclofenac for CYP2C9, and omeprazole for CYP2C19), TSAHC (0–20 µM), and human liver microsomes were preincubated for 5 min at 37°C. The final concentration of methanol in the incubations was 1.0%. The substrates were used for determination of IC50 values at the following concentrations: 1 µM for amodiaquine, 1 µM for diclofenac, and 10 µM for omeprazole. For the determination of Ks values, various concentrations of substrates (0.5–2 µM amodiaquine, 0.2–1 µM diclofenac, and 10–50 µM omeprazole) were used. After preincubation, the reactions were initiated by addition of an NADPH-generating system (1.3 mM NADP, 3.3 mM G6P, 3.3 mM MgCl2, and 500 unit/ml G6PDH) and stopped after 15 min by placing the incubation tubes on ice and adding 50 µl of ice-cold acetonitrile, containing an internal standard (10 mg/ml dextorphan). The incubation mixtures were centrifuged (10,000 g for 5 min at 4°C), and aliquots of the supernatants were analyzed by LC-MS/MS. The reaction rates were linear with incubation time and with microsomal protein content under these conditions.

**LC-MS/MS Analysis**

All metabolites of the P450 isofrom-specific substrates, excluding N-desethylamodiaquine, 4-hydroxydiclofenac, and 5-hydroxysomeprazole were determined by tandem mass spectrometry as described.
The system consisted of a Thermo TSQ Vantage Triple-stage quadrupole mass spectrometer (ThermoFischer Scientific, San Jose, CA, USA) equipped with an electrospray ionization interface used to generate positive ions [M+H]⁺. N-Desethylamodiaquine was separated on a HILIC column (Kinetex, 2.1 mm i.d. × 100 mm, 2.6 µm particle size; Phenomenex, Torrance, CA, USA) with an isocratic mobile phase consisting of acetonitrile and water [60/40 (v/v)] containing 0.1% formic acid. 4-Hydroxydiclofenac and 5-hydroxyomeprazole were separated on a Brownlee SPP C18 column (2.1 mm i.d. × 100 mm, 2.7 µm particle size; Phenomenex) and on a Luna C18 column (2.0 mm i.d. × 30 mm, 5 µm particle size; Phenomenex), respectively, with an isocratic mobile phase consisting of acetonitrile and water [80/20 (v/v)] containing 0.1% formic acid. The mobile phase was eluted using a Thermo Accela pump system consisting of acetonitrile and water [80/20 (v/v)] containing 0.1% formic acid. The mobile phase was operated at a flow rate of 0.2 ml/min. The mass spectrometer was operated in positive ionization mode and calibrated using the manufacturer’s calibration mixture. The operating conditions were as follows: capillary temperature: 350°C; vaporizer temperature: 300°C; ionization voltage: 4,000 V. The collision gas was nitrogen at a nominal pressure of 1.5 mTorr. The collision energy was set to 17, 20, 10, and 35 eV for N-desethylamodiaquine, 4-hydroxydiclofenac, 5-hydroxyomeprazole, and dextorphan, respectively. Quantitation was performed by selected reaction monitoring (SRM) of the precursor ion and the related product ion for each metabolite, using an internal standard to establish peak area ratios. The mass transitions used for quantitation of N-desethylamodiaquine, 4-hydroxydiclofenac, 5-hydroxyomeprazole, and dextorphan were m/z 328→283, 312→266, 362→214, and 258→157, respectively. The analytical data were processed using the Xcalibur software (version 2.1).

Data Analysis
The cytochrome P450-mediated activities in the presence of inhibitor were expressed as percentages of the corresponding control values in the presence of methanol alone. The apparent kinetic parameters for inhibitory potential (Kᵢ values) were initially estimated by graphical methods such as Lineweaver–Burk and Dixon plots, but ultimately determined by nonlinear least-squares regression analysis from the best enzyme inhibition model [24] using WinNonlin software (version 4.0; Pharsight, Mountain View, CA, USA). In this study, WinNonlin estimation consistently showed that inhibition data were best fitted by a noncompetitive inhibition model via Akaike information criteria and Schwartz criteria among the models tested, which included competitive inhibition, noncompetitive inhibition, and uncompetitive inhibition [23, 25].

**Fig. 2.** Inhibitory effect of TSAHC on P-glycoprotein-mediated digoxin efflux.

Cellular accumulation of 0.1 µM digoxin for 30 min in the absence and the presence of Cyclosporin A (CsA, 20 µM), p-aminohippuric acid (PAH, 100 µM), and TSAHC (1, 5, 20 µM) in LLCPK1-MDR1 cells. Data are derived from triplicate experiments.

**Table 1.** Inhibitory potency of TSAHC on specific P450 activities in pooled human liver microsomes (Gentest H161).

<table>
<thead>
<tr>
<th>Enzyme activity</th>
<th>P450</th>
<th>IC₅₀ (µM)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenacetine O-deethylation</td>
<td>1A2</td>
<td>&gt; 20</td>
</tr>
<tr>
<td>Coumarin 7-hydroxylation</td>
<td>2A6</td>
<td>&gt; 20</td>
</tr>
<tr>
<td>Bupropion hydroxylation</td>
<td>2B6</td>
<td>&gt; 20</td>
</tr>
<tr>
<td>Amodiaquine N-deethylation</td>
<td>2C8</td>
<td>1.0</td>
</tr>
<tr>
<td>Diclofenac 4-hydroxylation</td>
<td>2C9</td>
<td>0.08</td>
</tr>
<tr>
<td>Omeprazole 5-hydroxylation</td>
<td>2C19</td>
<td>4.5</td>
</tr>
<tr>
<td>Dextromethorphan O-deethylation</td>
<td>2D6</td>
<td>&gt; 20</td>
</tr>
<tr>
<td>Chloroxazone 6-hydroxylation</td>
<td>2E1</td>
<td>&gt; 20</td>
</tr>
<tr>
<td>Midazolam 1'-hydroxylation</td>
<td>3A</td>
<td>&gt; 20</td>
</tr>
</tbody>
</table>

* Averages of triplicate determinations.
Effects of TSAHC on CYP2C8, CYP2C9, and CYP2C19

As TSAHC showed inhibition of CYP2C isoforms, we sought to clarify the underlying mechanism. The inhibitory potential ($K_i$ values) of TSAHC on CYP2C isoforms was in the order CYP2C9 > CYP2C8 > CYP2C19 (Table 2). Lineweaver-Burk plots, Dixon plots, and secondary reciprocal plots indicated that TSAHC noncompetitively inhibited CYP2C8-, CYP2C9-, and CYP2C19-catalyzed oxidation, with apparent $K_i$ values of 0.81, 0.076, and 3.45 mM, respectively (Fig. 4, Table 2).

**DISCUSSION**

Using a well-validated *in vitro* model of drug metabolism based on human liver microsomes, high concentrations of TSAHC showed negligible inhibition of six human P450 isoforms (CYP1A2, CYP2A6, CYP2B6, CYP2D6, CYP2E1, and CYP3A). However, TSAHC noncompetitively inhibited three CYP2C isoforms based on a comparison of the inhibition constant ($K_i$) values. The estimated $K_i$ values of TSAHC on the CYP2C8-catalyzed amodiaquine N-deethylation, CYP2C9-catalyzed diclofenac 4-hydroxylation, and CYP2C19-catalyzed omeprazole 5-hydroxylation by TSAHC in pooled human liver microsomes (Gentest H161).

**Table 2.** $K_i$ values for the inhibition of CYP2C8-catalyzed amodiaquine N-deethylation, CYP2C9-catalyzed diclofenac 4-hydroxylation, and CYP2C19-catalyzed omeprazole 5-hydroxylation by TSAHC in pooled human liver microsomes (Gentest H161).

<table>
<thead>
<tr>
<th>Enzyme activity</th>
<th>P450</th>
<th>$K_i$ (µM)$^*$</th>
<th>Type of inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amodiaquine N-deethylation</td>
<td>2C8</td>
<td>0.81</td>
<td>Noncompetitive</td>
</tr>
<tr>
<td>Diclofenac 4-hydroxylation</td>
<td>2C9</td>
<td>0.076</td>
<td>Noncompetitive</td>
</tr>
<tr>
<td>Omeprazole 5-hydroxylation</td>
<td>2C19</td>
<td>3.45</td>
<td>Noncompetitive</td>
</tr>
</tbody>
</table>

$^*$Averages of triplicate determinations.

**Fig. 4.** Representative Dixon plots for the inhibition by TSAHC (0–20 µM) of CYP2C8-catalyzed amodiaquine N-deethylation (A), CYP2C9-catalyzed diclofenac 4-hydroxylation (B), and CYP2C19-catalyzed omeprazole 5-hydroxylation (C) upon incubation of pooled human liver microsomes (Gentest H161). Data are derived from triplicate experiments.
and CYP2C19-catalyzed omeprazole hydroxylation activities were 0.81, 0.076, and 3.45 µM, respectively (Table 2). The results demonstrate that TSAHC has selective inhibitory effects on CYP2C isoforms in vitro.

In general, various chemical inhibitors have been used in studies with human liver microsomes to elucidate the enzymes responsible for drug metabolism. For example, proadifen [4] and metyrapone [2] have been used as non-selective P450 inhibitors for the identification of the involvement of P450 isoforms in metabolite formation. In addition, chemical inhibitors, selective for specific P450 isoforms, are essential tools for determining the contributions of these enzymes to the metabolism of new chemical entities. Although potent specific inhibitors are available for each P450 isoform (for example, furafylline for CYP1A2, methoxsalen for CYP2A6, montelukast for CYP2C8, sulfaphenazole for CYP2C9, S-benzylthiranol for CYP2C19, quinidine for CYP2D6, and ketoconazole for CYP3A) [12], a compound that simultaneously inhibits three CYP2C-isoform activities without inhibiting the other P450 isoforms (CYP1A2, CYP2A6, CYP2B6, CYP2D6, CYP2E1, and CYP3A) is not available. Nicardipine has an inhibitory effect on three CYP2C isoforms (Kᵢ = 1.1–17.3 µM), but this drug also inhibits CYP2D6 and CYP3A with Kᵢ values of 2.9 and 1.6 µM, respectively [21]. Omeprazole inhibits both CYP2C9 (IC₅₀ = 40.1 µM) and CYP2C19 (IC₅₀ = 3.1 µM) [18], but not CYP2C8 [29], and therefore, cannot be used as a universal CYP2C inhibitor. TSAHC might present such a universal CYP2C isoform inhibitor, to elucidate the involvement of CYP2C isoforms in metabolism.

The energy-dependent efflux transporter P-gp is present in many tissues; it is found at the apical surface of mature enterocytes, the canalicular membranes of hepatocytes, and in kidney and brain endothelial cells [1]. Drugs that are substrates for transport by P-gp may have low oral bioavailability due to transport back into the gastrointestinal tract lumen [15]. Recently, increasing evidence suggests that some drug interactions result from changes in the activity and/or expression of drug transporters [8]. Impairment of P-gp-mediated transport is a well-known potential cause of drug interactions that enhance oral bioavailability [27]. In the present study, TSAHC increased digoxin accumulation in a dose-dependent manner in the LLCPK1-MDR1 cell system, suggesting that TSAHC has inhibitory effects on P-gp. However, this effect is less marked than that of CsA, which is one of the most commonly used P-gp inhibitors [16, 20]. It is important to note that inhibition of CYP2C and P-gp activities in vitro does not necessarily translate into drug interactions in vivo. Therefore, in vivo animal studies investigating the interactions between TSAHC and CYP2C and P-gp substrates are necessary to determine whether inhibition of CYP2C and P-gp activities by TSAHC is relevant.

Acknowledgments

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References


